The Bottlenose Dolphin (*Tursiops truncatus*) as a Model to Understand Variation in Stress and Reproductive Hormone Measures in Relation to Sampling Matrix, Demographics, and Environmental Factors

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Award Number: N000141110542
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LONG-TERM GOALS

Our overarching goal is to develop indicators and methods to quantify chronic stress in bottlenose dolphins. Much research has focused on the stimuli which induce stress in marine mammals, as well as the hormonal mediators of the stress response. Stress may be induced by a variety factors, including noise, pollutant or toxin exposure, presence of predators, loss of prey, and/or habitat changes. The stress response is complex and difficult to study experimentally in marine mammals due to ethical and logistical considerations, but has been well characterized in other laboratory mammal species. In mammals (including marine mammals) and other vertebrates, the stress response has two modes of operation: 1) the fast mode involves the rapid release of fast-acting agents that drive the fight-or-flight response, such catecholamines, which excite the hypothalamic-pituitary-adrenal (HPA) axis and initiates a hormonal cascade that ends in the secretion of glucocorticoids (GCs) by the adrenal cortex; and 2) the delayed but more sustained response driven by GCs that coordinates brain and body functions to cope with stress and facilitate recovery, adaptation, and re-establishment of homeostasis.

While the HPA axis and physiological processes driven by the GCs are essential for an individual's ability to respond and adapt to stress, prolonged elevation of GC hormones can lead to chronic immune suppression and inhibition of other energy expending hormonal systems, including disruption of reproductive function along the HPA axis, all of which may cumulatively lead to decreased survival and/or inability to reproduce. For this reason, developing indicators and methods to quantify chronic stress in marine mammals is essential for understanding risks and long-term consequences for populations.

OBJECTIVES

Using the bottlenose dolphin as a model species, specific objectives for this project are:

Report Documentation Page

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
30 SEP 2013		**00-00-2013 to 00-00-2013**

4. TITLE AND SUBTITLE	5a. CONTRACT NUMBER
The Bottlenose Dolphin (Tursiops truncatus) as a Model to Understand Variation in Stress and Reproductive Hormone Measures in Relation to Sampling Matrix, Demographics, and Environmental Factors	5b. GRANT NUMBER
	5c. PROGRAM ELEMENT NUMBER
6. AUTHOR(S)	5d. PROJECT NUMBER
	5e. TASK NUMBER
	5f. WORK UNIT NUMBER
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) **Chicago Zoological Society,c/o Mote Marine Lab,1600 Ken Thompson Parkway,Sarasota,FL,34236**	8. PERFORMING ORGANIZATION REPORT NUMBER
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)	10. SPONSOR/MONITOR'S ACRONYM(S)
	11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

15. SUBJECT TERMS

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			
unclassified	**unclassified**	**unclassified**	**Same as Report (SAR)**	**6**	

1. Determine correlation of hormone measures between blood and blubber
2. Develop a comprehensive understanding of factors that influence stress hormone levels and establish reference intervals, determining necessary stratifications by sex, age and/or sampling season.
3. Examine relationships among the various hormone measures, and conduct preliminary screening analysis to examine potential relationships between the stress hormones and other health measures including immune function.

APPROACH

Field Studies

The Chicago Zoological Society's (CZS) "natural laboratory" situation in Sarasota Bay, Florida provides unique opportunities to address questions related to stress, as a resident population of bottlenose dolphins has been studied for more than 40 years, relying upon methods which include capture-release health assessments. Stress and reproductive hormones (cortisol, aldosterone, thyroid, testosterone, progesterone) have been routinely measured in blood serum as part of the health assessment, which also includes a complete physical examination, morphometric measurements, hearing tests, and biological sampling of skin, blubber, urine, feces, gastric contents, and blowhole. These tissue samples are analyzed for a broad suite of diagnostics. We have leveraged this existing collection of data and also conducted additional sampling during Sarasota Bay health assessments over the past 3 years to help meet our proposed objectives. We have also included existing data from prior (2009) capture-release health assessments along the Georgia coast.

In addition, recent work by our collaborative team has supported the use of blubber as a sampling matrix for measuring hormone concentrations. Blubber provides an advantage in that samples can be readily obtained using remote biopsy, which is relatively inexpensive and entails less harassment compared to capture-release sampling. In addition, while the logistics of capture-release sampling generally limit its utility to investigations of coastal cetaceans, remote biopsy has the potential to be a powerful tool for investigations across a range of habitats, from estuarine to nearshore and pelagic populations. To elucidate potential seasonal variation in blubber hormone measures, remote biopsy samples have been collected across 4 seasons in estuarine and coastal waters near Charleston, South Carolina, and across 2 seasons in the Ashepoo, Combahee and Edisto (ACE) Basin, also in South Carolina.

Laboratory Analyses

Hormone concentrations (cortisol, aldosterone, reproductive and thyroid hormones) in serum samples have been analyzed by Cornell Animal Health Diagnostic Center (AHDC) Endocrinology Laboratory. Cortisol and reproductive hormone concentrations in matched blubber samples from the capture-release health assessments have been analyzed by Nick Kellar's laboratory using commercially available enzyme-immuno (EIA) assay kits (Kellar et al. 2006, Kellar et al. 2009).

For the analysis of the remote biopsy blubber samples, we have begun collaboration with Dr. Ashley Boggs (National Institute of Standards and Technology) and Dr. Louis Guillette (Medical University of South Carolina). Dr. Boggs is in the process of developing and

validating a new method using solid phase extraction (SPE) to liquid chromatography tandem mass spectrometry (LC-MSMS) to extract and directly quantify multiple classes of hormones from a single sample. Once Dr. Boggs has completed validation of this method, she will perform the analysis of our remote biopsy samples, thus providing us information on a much broader suite of hormones for our samples.

Data Analyses

Objective 1- In efforts to investigate the dynamics of cortisol in different dolphin tissues, cortisol measurements from blubber and blood were modeled relative to handling time in live-captured individuals. Handling time was defined as the time from when the capture net was released for encirclement until the time that the tissue was sampled. Each individual used in this analysis was represented by paired blubber and blood measurements with varying handling times, but in each case blood collection preceded blubber collection.

Objectives 2-3 - Statistical methods were used to evaluate demographic and sampling factors contributing to observed differences in serum concentrations of adrenal and thyroid hormones (i.e. cortisol, aldosterone, T3, T4, FT4), and relationships among these various hormones in bottlenose dolphins sampled (via capture-release methods) in Sarasota Bay, Florida, USA (2000-2012). While it is helpful to understand demographic and environmental influences on endocrine hormone concentrations among individuals and across populations, the impact of various biological and artificial stressors cannot be evaluated without a comparison to baseline "normal" values. Results of the statistical tests to identify factors influencing hormone concentrations and nonparametric bootstrap methods (Schwacke et al. 2009) were used to develop stratified 90^{th} and 95^{th} percentile reference intervals for each hormone constituent.

The project is a collaborative effort led by Dr. Lori Schwacke (NOAA/National Ocean Service (NOS)/National Centers for Coastal Ocean Science (NCCOS)) and Dr. Randall Wells (Chicago Zoological Society). Other collaborators and co-PIs are Eric Zolman, NOAA/NOS/NCCOS, Dr. Nicholas Kellar, NOAA/National Marine Fisheries Service (NMFS), Southwest Fisheries Science Center, Dr. Patricia Rosel, NOAA/NMFS Southeast Fisheries Science Center, Dr. Stephanie Venn-Watson, National Marine Mammal Foundation, Dr. Teri Rowles, NOAA/NMFS, Office of Protected Resources, Dr. Leslie Hart, NOAA/NOS/NCCOS, and Dr. Ashley Boggs, NIST.

WORK COMPLETED

All of the planned capture-release and remote biopsy fieldwork has been completed. Matched blood and blubber samples were collected from dolphins in Sarasota Bay during May 2009 (n=20), May 2010 (n=10), May 2011 (n=15), and May 2012 (n=16). Additional blood samples, without blubber, were collected in July 2012 (n=10). More than 2,100 hormone measures previously obtained for Sarasota Bay dolphins are being applied to this project. Remote biopsy sampling to collect blubber samples across seasons was conducted during Fall 2011 and Winter, Spring, Summer 2012 in waters near Charleston, SC, and conducted in the ACE Basin during Winter and Summer 2012. In total, 118 blubber samples were collected for hormone analysis.

Objective 1 - We began comparing cortisol concentrations between blood and blubber with handling time as a covariate using a modeling process composed of three stages: 1) model

selection for the blood measurements, 2) model selection for the blubber measurements, and 3) estimation of the length of time that the relative values in the blubber lag those in the blood at different stages of each tissue's dynamic progression. For model selection, only sigmoidal functions were tested; the rationale was based in first principles that there is a limit to maximum sustained cortisol production by the adrenal glands (Marik and Zaloga 2002) and that if sustained for a sufficient duration it will reach equilibrium (dynamic or otherwise). The functions tested included standard logistic, generalized logistic variants (using 4 - 6 parameters), Michaelis–Menten, Gompertz, and Von Bertalanffy structures. Deviance information criterion (DIC), a Bayesian analog to Akaike information criterion, was used to assess relative fit among the different models.

Objectives 2-3 - For all stress and thyroid hormones (cortisol, aldosterone, T3, T4, and Free T4), correlational tests, ANOVA, Mann Whitney U tests, and Generalized Linear Mixed Models (GLMM) were used to determine associations between each individual hormone and demographic/sampling parameters. Demographic variables included age, sex, maturity status, length, mass, and lactation status, and sampling variables involved sampling year, handling time, and the time at which samples were collected (sampling time of day). Observations used for analyses were limited to May-July, when most of the sampling occurred, to avoid seasonal confounding. Furthermore, data were limited to the years 2000-2012 to maintain laboratory consistency. Pregnant animals, identified by ultrasonography, were also excluded from analyses because of hormonal fluctuations that can occur with pregnancy. All individuals with known ages were categorized based on sexual maturity status to evaluate the influence of maturity status on hormone concentrations.

Nonparametric bootstrap methods were used to estimate 90^{th} and 95^{th} percentile reference intervals and corresponding 90% confidence intervals for cortisol, T3, T4, and FT4 (Schwacke et al. 2009). Hormone data for the reference intervals were stratified according to significant covariates determined by the GLMMs, and the need for data partitioning was evaluated using bootstrap methods and confidence interval calculations according to procedures described by Schwacke et al. (2009).

RESULTS

Objective 1 - A linear regression analysis between blood and blubber values indicated a statistically significant positive relationship between cortisol values in blood and blubber ($p < 0.0001$, $r^2 = 0.59$, $n = 49$). The fit of several nonlinear models was evaluated using DIC values, and the 4 parameter logistic was selected to estimate the lag in cortisol concentration in the blubber as compared to the blood. We estimated that at 25% of the maximum modeled value, the blubber values lagged those in the blood by 71mins. At 50% the lag was 98mins, at 75% it was 136mins and at 99% it 190mins (Fig. 1). These results indicate that blubber cortisol may mimic the levels in the blood but lag by 1-3 hours. Assuming that the decrease in cortisol follows a similar course, this suggests that blubber cortisol is representative of the integration of blood cortisol levels for a few hours to a half day before sampling. It also suggests that when biopsying, for example, the increase in cortisol due to the act of sampling has little effect on blubber cortisol levels (assuming that action occurs within minutes to tens of minutes).

Therefore, remote biopsy sampling may provide a good measurement of baseline cortisol in free ranging bottlenose dolphins.

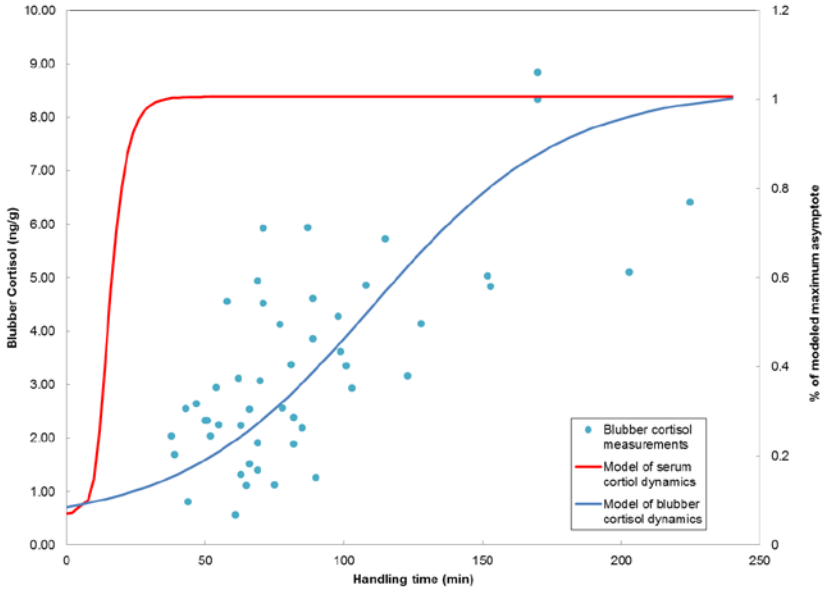

Figure 1. Dynamics models of blood and blubber values relative to handling time. The solid lines represent the best fit models for blood (red) and blubber (blue) relative to % of the modeled maximum asymptotes (provides a standardized unit for comparison). The blue circles represent the blubber cortisol measurements. The blood cortisol measurements were not included to minimize visual complexity.

Objectives 2-3 - The 2000-2012 dataset used for these analyses included 185 observations representing 121 individuals (61 males, 60 females). The ages of dolphins sampled during this time period spanned 2-50 years (males: 2-43; females: 2-50), providing an opportunity to examine hormone concentrations across a broad age range. Cortisol concentrations were significantly associated with handling time and mass, and reference intervals were stratified by handling time (<30 min and ≥ 30 min). Progesterone was positively correlated with cortisol, even among male dolphins, suggesting that progesterone may also be involved in the stress response. T3 and FT4 reference intervals were stratified by maturity status. TT4 concentrations were associated with sex and maturity status, resulting in reference intervals stratified by both sex and maturity status.

Dr. Hart has prepared a draft manuscript summarizing our results to date under this objective and it is currently being reviewed by co-authors.

IMPACT/APPLICATIONS

Our results to date provide tremendous new insight into the dynamics of cortisol in both blood and blubber for dolphins experiencing acute stress, as well as provide critical baseline information on cortisol and other hormone concentrations in wild dolphin populations. This understanding is absolutely essential for future stress studies in dolphins and other cetaceans in

order to appropriately interpret stress hormone measures. Furthermore, the reference intervals produced by this study provide the basis for evaluation of animal health that is necessary for future population assessments.

RELATED PROJECTS

A matching project is being conducted under the leadership of Dr. Lori Schwacke of the NOAA/National Ocean Service Hollings Marine Laboratory (Project No. N0001412IP20053). Data analyses are being performed jointly.

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